S-ETHYL-COENZYME A AND ACETONYLDETHIO-COENZYME A

Interactions with pyruvate carboxylase and phosphotransacetylase

H. P. BLASCHKOWSKI, J. KNAPPE and Th. WIELAND*

Institut für Biologische Chemie, Universität Heidelberg, Neuenheimer Feld 501, D-6900 Heidelberg and **Max-Planck-Institut für Medizinische Forschung, Abteilung Naturstoff-Chemie, Jahnstrasse 29, D-6900 Heidelberg, FRG*

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1. Introduction

Acetonyldethio-coenzyme A, an analogue of acetyl-CoA with the sulfur being replaced by methylene, has been shown to interact with citrate synthase [1] and with acetyl-CoA carboxylase [2] as a competitive inhibitor and a substrate, respectively. When extending these studies to further enzymes which bear an acetyl-CoA binding site it appeared interesting to test also *S*-ethyl-CoA to thus obtain an indication of both the roles of the sulfur as well as the carbonyl-oxygen in the recognition of acetyl-CoA. *S*-Ethyl-CoA and homologous *S*-alkyl-CoA compounds were conveniently prepared from CoA by reaction with the respective alkyl bromides. This paper reports the effects of the various analogues on the regulatory site of pyruvate carboxylase and on the catalytical site of phosphotransacetylase.

2. Experimental

2.1. S-*Methyl-*, S-*ethyl-*, S-*propyl- and* S-*butyl-CoA*

The solution of 50 mg CoA (Boehringer Mannheim) and 100 μmol dithiothreitol in 5 ml water was flushed with argon, adjusted to pH 9–10 with 1 M LiOH and reacted at 20°C either with 40 μl dimethyl sulfate or with an ethanolic solution (10 ml) of 0.4 ml ethyl, propyl or butyl bromide. After 2 h standing at pH 9–10 the mixture was cooled to 4°C and diluted with 200 ml 3 mM HCl and applied to a DEAE-cellulose column (DE 52, Whatman; 2.5 × 20 cm) pre-equilibrated with 3 mM HCl and eluted by a linear gradient (1000 ml) from 0–0.15 M LiCl in 3 mM HCl. The fractions containing alkyl-CoA (0.09–0.12 M LiCl) were adjusted to pH 7 with 1 M LiOH and lyophilized, LiCl was extracted with acetone/methanol (95/5). The residue was dissolved in a minimum amount of water, precipitated with acetone/methanol and dried in vacuo, yielding about 30 mg.

In paper electrophoresis (ammonium acetate, pH 4.5) and in ascending paper chromatography (butanol/acetic acid/water (5/2/3)) each preparation showed a single spot in ultraviolet and upon spraying for phosphate and sulfur containing compounds. The ratio of adenine (ϵ_{260} = 16 000 $M^{-1}cm^{-1}$) : phosphate (Fiske-Subbarow assay) : thioether (assay according to [3], using *N*-acetyl-*S*-ethylcysteamine as reference) was determined for ethyl-CoA to 1:3.06:1.04.

The proton-decoupled natural abundance ^{13}C NMR spectrum (recorded with a Bruker HF-X90 instrument at 22.63 MHz in the pulsed Fourier transform mode) of ethyl-CoA in D_2O was consistent with that of CoA (cf. [4]) except for the *S*-ethylcysteamine moiety $CH_3-CH_2-S-CH_2-CH_2-NH-$:

δ (ppm downfield from tetramethylsilane) 15.2, 26.2, 31.1 and 39.8.

2.2. N-*Acetyl*-S-*ethylcysteamine*

This reference compound was prepared from 85 mmol *N*-acetylcysteamine [5] and 15 ml ethyl bromide in 300 ml ethanol/water (50/50) at 15°C keeping the mixture at pH 10 by addition of 10 M KOH. When –SH had disappeared after 1 h, the mixture was neutralized with sulfuric acid, and

solvents were evaporated under reduced pressure. The residue was extracted with chloroform and fractionated by destillation in vacuo, yielding 8 g pure compound as a colourless liquid; b.p. 115–116°C, 1 mm. (Found: C, 48.74; H, 9.06; N, 9.26; S, 21.53. Calculated for $C_6H_{13}ONS$: C, 48.95; H, 8.90; N, 9.51; S, 21.77.)

^{13}C NMR in D_2O (*S*-ethyl-cysteamine moiety, see section 2.1.): 15.1, 26.4, 31.3, 40.1 ppm.

2.3. *Other chemicals and enzymes*

Acetyl-CoA was prepared enzymatically from acetyl phosphate (500 μmol) and CoA (50 μmol) using phosphotransacetylase (0.1 mg); it was subjected to DEAE-cellulose chromatography as in section 2.1. and determined according to [6]. Acetonyldethio-CoA was synthesized as in [1] and determined spectrophotometrically by assuming ϵ_{260} = 16 400 $M^{-1}cm^{-1}$. All other substrates and chemicals were of highest quality available. Phosphotransacetylase (*Clostridium kluyveri*) malate dehydrogenase and citrate synthase were from Boehringer Mannheim. Pure pyruvate carboxylase was isolated from chicken liver by the procedure in [7].

2.4. *Enzyme assays*

Pyruvate carboxylase activity was measured by the optical assay [8] at 25°C. The mixture (1 ml) contained 100 mM Tris–HCl (pH 7.8), 10 mM Na-pyruvate, 1 mM ATP, 5 mM $MgCl_2$, 15 mM $KHCO_3$, 3.2 mM $(NH_4)_2SO_4$ (cf. [7]), 0.3 mM NADH, varying concentrations of acetyl-CoA analogues, 5 μg malate dehydrogenase and 10 μg pyruvate carboxylase.

Phosphotransacetylase activity in the forward reaction (as employed in the experiments of table 2) was measured following acetyl-CoA conversion to CoASH which was determined colorimetrically with Ellman's reagent. The assay mixture (2 ml) contained 200 mM K-phosphate (pH 7.5), varying concentrations of acetyl-CoA and analogues and (routinely) 0.08 μg enzyme. At 2 min, 4 min and 6 min incubation at 25°C 0.5 ml samples were withdrawn and admixed to 0.05 ml 10 mM 5,5′-dithiobis(2-nitrobenzoate); the color was recorded at 405 nm. Mean values of the enzyme reaction rate were computed using a molar absorbance of 13 500 $M^{-1}cm^{-1}$. With 1 mM acetyl-CoA, the specific activity of the enzyme employed was 148 μmol min^{-1} mg^{-1}.

For the experiments of fig.2, phosphotransacetylase activity was determined by a coupled optical assay. The mixture (1 ml) contained 50 mM Tris–HCl (pH 8), 0.1 M KCl, 3 mM dithiothreitol, 10 mM acetyl phosphate, 0.1 mM CoA, 1.2 mM NAD, 5 mM DL-malate, 25 μg malate dehydrogenase, 20 μg citrate synthase and 100 μg bovine serum albumin.

3. Results and discussion

Pyruvate carboxylase from avian liver is virtually totally dependent on acetyl-CoA for catalytical activity (cf. [9]). The data in fig.1 and table 1 show that ethyl-CoA and acetonyldethio-CoA both could fully replace acetyl-CoA. Also, the activation parameters (half-activation constant, Hill coefficient)

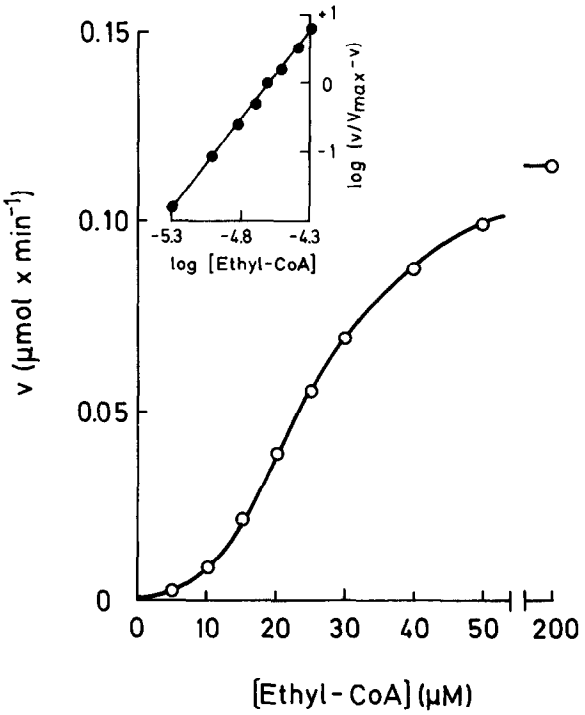

Fig.1. Effect of ethyl-CoA on pyruvate carboxylase activity. Enzyme activity was measured at pH 7.8 and 25°C by the coupled optical assay [8] with concentrations of ethyl-CoA as indicated. The amount of enzyme was 10 μg. The inset represents the plot of the values according to the empirical Hill equation from which n_H and K were obtained.

Table 1
Activation of pyruvate carboxylase by acetyl-CoA analogues

Compound	K (μM)	n_H	V_{max} (%)
Acetyl-CoA	4.1	2.8	100
Acetonyldethio-CoA	57	2.1	100
Ethyl-CoA	25	2.6	100
Methyl-CoA	270	2.0	40
Propyl-CoA	32	1.9	97
Butyl-CoA	190	1.8	61

The kinetic parameters are for pH 7.8 (25°C) and were determined as outlined in fig.1. K values are the activator concentrations which produce half-maximum effect. For the same conditions as used here K and n_H values were reported [7] for acetyl-CoA as 3.3 (μM) and 3.0, respectively

Table 2
Competitive inhibition of phosphotransacetylase by acetyl-CoA analogues

Compound	K_i (mM)
Acetonyldethio-CoA	0.36
Methyl-CoA	1.7
Ethyl-CoA	1.0
Propyl-CoA	1.8
Butyl-CoA	2.8

Enzyme activity was measured in the forward reaction from acetyl-CoA at pH 7.5 and 25°C (see section 2). Varying concentrations of acetyl-CoA (K_m 0.32 mM) and analogues were employed and K_i values were computed from Dixon plots

of these compounds were quite similar to those of the natural activator.

Methyl-CoA and butyl-CoA showed markedly diminished effectiveness (table 1). This is consistent with the results from studies of a large variety of acyl-CoA compounds [9], that a chain of two carbon atoms is optimally accomodated by the acyl-binding part of the regulatory site of the avian liver enzyme. This interaction is obviously predominantly hydrophobic and should not involve an essential hydrogen bond, since the carbonyl-oxygen (and sulfur) of acetyl-CoA is dispensible for initiating the allosteric transition of the protein.

Phosphotransacetylase from *C. kluyveri* was expectedly found to be reversibly inhibited by the analogue compounds employed. They acted as competitive inhibitors with respect to acetyl-CoA at a saturating concentration of phosphate. The K_i-values are summarized in table 2.

An essential –SH group has been detected in the catalytical site of phosphotransacetylase [10]. It is protected by acetyl-CoA (or acetyl phosphate, but not by desulfoCoA) against reaction with *N*-ethylmaleimide. The same protecting effect could also be provided by ethyl-CoA and methyl-CoA (other analogues have not been tested) and was better than 95% at 'saturating' concentrations (20 mM). Figure 2 shows the experiment with ethyl-CoA. The reduction of the rate of enzyme inactivation by 50% was computed from these data to occur at 0.9 mM which represents the enzyme–ethyl-CoA complex K_d and is consistent with the K_i value determined for ethyl-CoA.

From this and the work with several acetyl-CoA dependent enzymes [1,2] it is evident that replacements of either the sulfur or the carbonyl group by

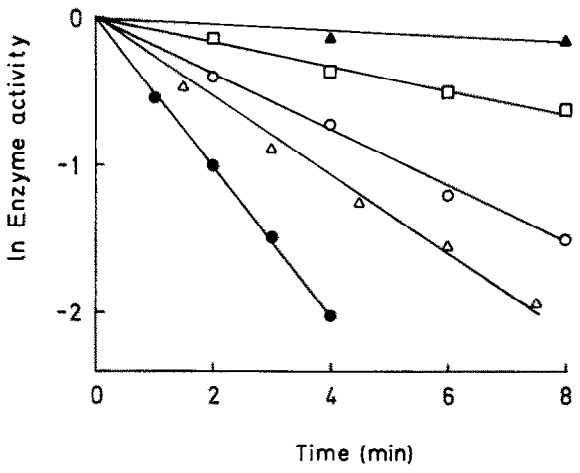

Fig.2. Protection by ethyl-CoA of phosphotransacetylase against inactivation by *N*-ethylmaleimide. The enzyme (20 μg/ml) was incubated at 25°C (0.1 M Tris–HCl (pH 8) plus 0.1 M KCl) with 0.1 mM *N*-ethylmaleimide and concentrations of ethyl-CoA (mM) as follows: (●) 0; (△) 0.8; (○) 1.6; (□) 4.8; (▲) 20. Aliquots of 20 μl were withdrawn and immediately admixed to the coupled optical enzyme assay (see section 2) at the time intervals shown. The logarithm of the fraction of residual activity was plotted versus time; the slopes show pseudo first-order rate constants of enzyme inactivation.

methylene do not significantly alter the specific binding properties of acetyl-CoA. For the many purposes where acetyl-CoA (or long chain acyl-CoA) analogues may be employed in biochemical studies, it should be advantageous to use the conveniently available and stable *S*-alkyl-CoA compounds.

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